DEVELOPMENT OF NUCLEIC ACID APTAMER-BASED SENSORS FOR THE DIRECT DETECTION AND IDENTIFICATION OF BIOLOGICAL AGENTS

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The objective of this work is to develop a novel sensing platform for the direct and rapid detection of biological agents. To achieve this goal, we are building on our portable DNA Biological Detection System work by incorporating a new class of molecular recognition elements, known as aptamers. In this paper, the nucleic acid-based sensor work conducted in our laboratory is discussed including initial work on the design and optimization of aptamer biorecognition sensor elements targeting a relevant biological agent surrogate (i.e., *Bacillus globigii*) and the development of a portable nucleic acid detection system.

INTRODUCTION

Current detection capabilities for biological warfare agents either provide rapid, early warning triggers that lack specificity, or specific and sensitive detection but require very long total analysis times and have high logistic loads. Another significant limitation of the currently fielded technologies is the inability to detect and identify the full spectrum of biological and chemical threats (i.e., chemical agents, toxins, viruses, bacteria, spores, etc.) on a single detection platform. For example, in DNA hybridization-based analyses and fluorescence-based immunoassays, speciation and sensitive analyses are possible, but often involve several time-consuming washing and binding steps.[1-2] Molecular beacons (MB), have been developed as an alternative to conventional DNA hybridization schemes, without requiring any washing and labeling steps.[3-4] However, despite this advance DNA hybridization methods require a cell-rupturing step to isolate the genetic material. This can be particularly difficult for some target agents such as *B. anthracis*, as it is very difficult to disrupt the strong, resistant shell of the spore.[5] Immunological or antibody-based methods do not require a cell-rupturing step since the target agent can be identified through specific ("lock and key") surface recognition sites.[2] However, the difficulty in obtaining antibodies through animal hosts and instability of antibody reagents significantly hampers immunological detection technique performance under battlefield and other field conditions.

Recently, a new class of molecular recognition elements, known as aptamers, has shown great potential for specific binding to a wide range of targets while overcoming many of the problems encountered with traditional biorecognition elements such as the poor stability and mass production capabilities of antibodies and the cell rupturing steps of DNA-based identification.[6-7] Aptamers are functional oligonucleotide strands that are selected from a random sequence pool of nucleic acids, for their specific binding to a variety of molecular targets. Recent applications have shown the selective affinity of aptamers for small organic molecules, amino acids, oligosaccharides, proteins (e.g., thrombin, Ricin), and even whole cells (e.g., red blood cells). [6-7] Typically aptamers are comprised of 15 to 60 nucleic acids, which fold into distinct three-dimensional structures, upon the selective association with their target ligands to form stable complexes. The affinities at which aptamers frequently bind with their

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targets (i.e. K_d = 1pM -1μM) rival that of monoclonal antibodies. The basis for the high binding affinities is due to an intricate, partial encapsulation of the target species and a variety of interactions within the aptamer-target complex including stacking, shape complementarity, electrostatic interactions, and hydrogen bonding. [6-7] Since nucleic acid aptamers have been shown to exhibit a wide range of recognition capabilities, the proposed new class of molecular probes incorporating a molecular beacon design, has great potential for real-time monitoring of various types of target threat agents ranging from viruses, toxins, bacteria and bacterial endospores.

EXPERIMENTAL METHODS

RANDOM OLIGONUCLEOTIDE LIBRARY

A random oligonucleotide library for aptamer screening containing known primer sequences flanking a randomized 40-base sequence was commercially synthesized for this work. This randomized library, 5'-GATGAGTTCGTGTCCGTACAACTGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCGCTGTGGCTGATTTCGATAACC-3'included a biotin label with a 12 atom linker on the 5' region of the randomized sequences to allow for detection of selected sequences during the screening process. Also synthesized were the primer sequences necessary for polymerase chain reaction (PCR) amplification. The first primer sequence synthesized was 5'-GATGAGTTCGTG TCCGTACAACTG G -3'. The second primer, 5'-GGTTATCGAAATCAGCCACAGCGCC -3' was synthesized with a 5' biotin label. All oligonucleotides were purified using reverse phase HPLC prior to use.

PCR AMPLIFICATION

A lambda phage DNA positive control (Applied Biosystems N801-0055) was amplified using the same oligonucleotide primers generated for the aptamer selection process. Optimized PCR amplification conditions used for both the positive control amplifications and the random aptamer library included an initial denaturing step at 94 ˚C for 5 minutes followed by 25 thermocycles of (1) 94 ˚C for 5 minutes denature step, (2) 68 ˚C anneal step, and (3) 72 ˚C extension step. The final step of the PCR protocol was a 72˚C extension for 7 minutes.

GEL ELECTROPHORESIS

Gels were prepared containing 2% Agarose gel and ethidium bromide for DNA staining. All gel electrophoresis experiments were achieved using a 40mM Tris, 20 mM Acetate, 2mM EDTA buffer in a 10 cm Minicell system (ThermoEC) operating at 117 V.

APTAMER SELECTION

The random oligonucleotide library was heated to ~ 96°C and then mixed with a suspension of the *Bacillus globigii* spores in sterile binding buffer (~1M NaCl, 40mM Tris-HCl, and 2mM $MgCl_2$). After incubating for an hour at room temperature, the suspensions were centrifuged to pellet the spores, and the unbound fraction (supernatant) was discarded. The spores were resuspended in heated (~95°C)deionized water and centrifuged to pellet the spores. The supernatant, containing the selected aptamers were then amplified using the previously determined optimized PCR conditions with a portion of the amplified product being monitored using gel electrophoresis as discussed above.

DNA HYBRIDIZATION EXPERIMENTS

Conventional DNA hybridization experiments were conducted using tapered fiber optic sensors. For this work, multimode, polymer-clad fibers were obtained from ThorLabs, Inc. The polymer jacket was mechanically stripped from the fiber and the cladding was removed by dissolving in acetone. The fiber

core was then cleaned by soaking in 25% nitric acid for approximately 30 minutes followed by extensive rinsing with distilled, deionized water (DDI H_2O). Once cleaned, the fibers were then tapered in a Sutter Instruments P-2000 laser-based fiber puller. Pull parameters such as velocity, force, heat and raster width were all adjusted for the appropriate fiber geometry. The fibers were generally 400 micron diameters tapered to single microns in a conical geometry over a 4 millimeter length.

Tapered and cleaned fiber tips were soaked in 2% aminopropyltriethoxysilane in DDI H_2O for 1 hour forming a monolayer with terminal amines for further modification. The fibers were rinsed thoroughly in DDI H_20. After rinsing, the fibers were heated to approximately 105 °C overnight to anneal and stabilize the monolayer. After cooling the fibers to room temperature, they were soaked in 2% glutaraldehyde in 1x Saturated Sodium Citrate Buffer (SSC) for 1 hour and then rinsed in 6xSSC. Finally, the fibers were placed in a solution (2 micrograms per milliliter) of amino-terminated capture oligonucleotide for at least 4 hours at 4 °C.

The tapered fiber tips were stored in the oligonucletide coating solution at 4 C until use. The fibers were removed and rinsed in 6xSSC and then placed in a test solution of labeled oligonucleotide for several minutes depending on the analyte concentration being investigated in order to insure 100% hybridization of viable surface bound capture probes. The fibers were then rinsed in 6xSSC to remove nonspecific binding and placed in the optical train and interrogated. The dye (IRD 800 from Licor, excitation maximum 787 nm, emission maximum 812nm) was selected in order to utilize an inexpensive laser source and avoid background fluorescence. The fluorescence was monitored with an experimental setup discussed in previous work using a 785 nm excitation laser and ocean optics spectrometer.[8]

PORTABLE DNA DETECTION SYSTEM

The portable DNA detection system used in this work was developed in conjunction with Echo technologies, Inc. The 1st generation prototype contains an integrated fluidics module, temperature controlled flow cell, fluorescence detection system and touch screen display interface.

RESULTS AND DISCUSSION

APTAMER SELECTION

The screening of high affinity aptamers is accomplished through the systematic evolution of ligands by exponential enrichment (SELEX) process, shown in Figure 1. SELEX is a versatile, combinatorial approach that begins with an initial random oligonucleotide library typically greater than 20 bases in length, each of which is flanked on both ends by known primer sequences, as shown in Figure 1. Unlike other combinatorial techniques, the size of the nucleic acid-selection population can be easily manipulated with the maximum diversity approximately equal to 4^n, where n is the length of the random portion of the sequence. Not surprisingly, the greater degree of diversity in the initial random library, the greater the opportunity to select for a very high affinity aptamer. [6-7]

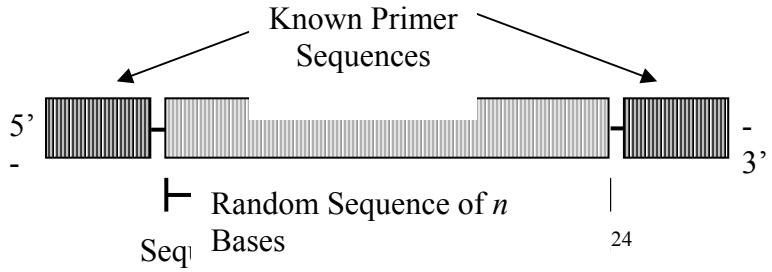

Figure 1. Random oligonucleotide library.

As illustrated in Figure 2, high binding affinity aptamers can be selected from the large random pool, through exposure to the target species. Sequences that readily bind are retained, and amplified with polymerase chain reaction (PCR) amplification using the known portions of the sequence for the binding of PCR primers. The process is repeated several times, to produce a set of high affinity aptamers.[6-7] In general, the greater the number of rounds of selection, the higher the binding affinity of the resulting aptamer to the target species. In addition, the selection process can be performed in-vitro, and mass production of the selected aptamers can be performed with an oligonucleotide synthesizer and PCR amplifier. Therefore, producing large quantities of aptamers for various BW agents is greatly simplified compared to immunological processes, which require antibody harvesting and purification from live animals.[6-7]

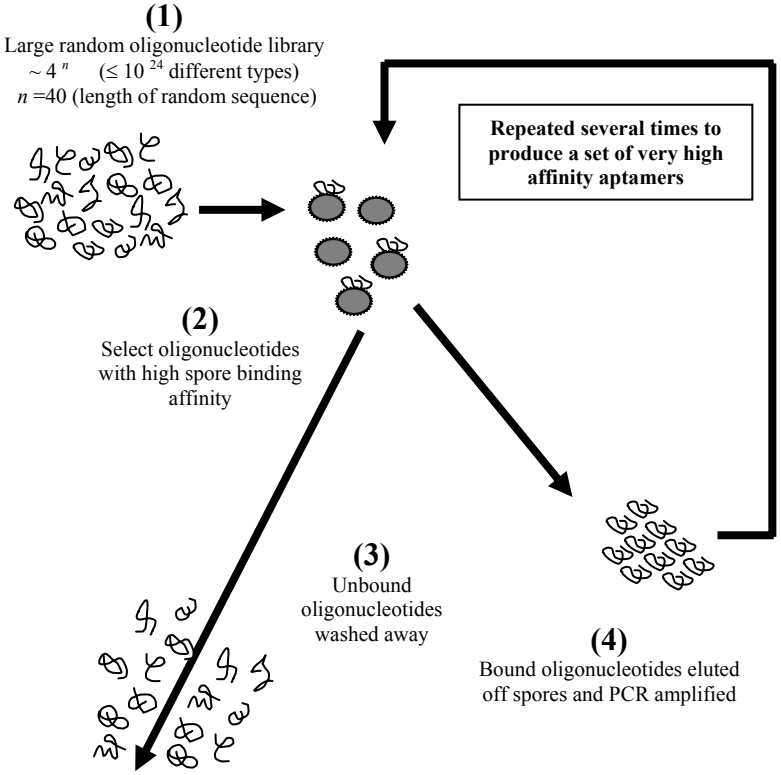

Figure 2. Aptamer selection process.

Prior to performing the optimization of the SELEX aptamer library, the polymerase chain reaction (PCR) amplification protocol was developed. In particular, the annealing temperature conditions are critical to the PCR yield, as the PCR primer sequences may encounter a partially complementary sequence in the aptamer, and may hybridize if proper temperature stringency is not met. Another potential concern is that partial internal hybridization within the randomized regions can also be self-priming for the Taq polymerase, thereby producing higher molecular weight PCR products. In order to monitor the molecular weight of the amplified oligonucleotide sequences, each successive selection and amplification steps are monitored through standard agarose gel electrophoresis as shown in Figure 3. For reference, lanes 1-2 contain molecular weight standards of known size (base pairs), and concentration. Lane 4 contains a positive control at 500 base pairs indicating successful PCR amplification of a control. Lanes 9-11 represent three successful PCR amplifications from a randomized aptamer library containing a sequence length of 90 base pairs.

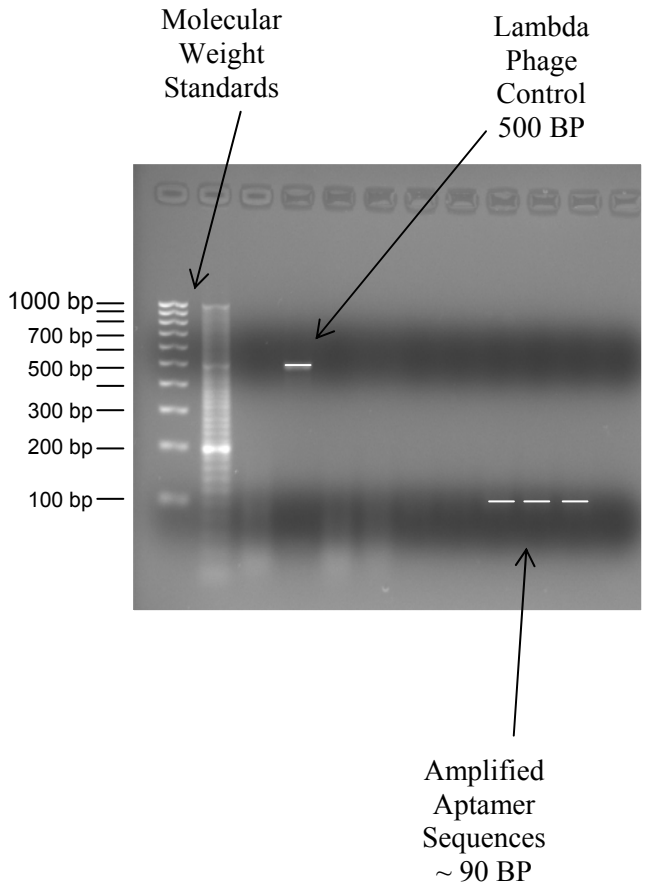

Figure 3. Gel electrophoresis of PCR amplified aptamers.

PORTABLE DETECTION SYSTEM

In order to evaluate the performance of the nucleic acid sensors, a fluorescence measurement system, capable of real-time measurements was constructed. A photograph of the 1st generation portable DNA system originally designed for the tapered DNA hybridization work conducted in our laboratory is shown in Figure 4.

Figure 4. Portable DNA detection prototype built with Echo Technologies, Inc.

This integrated device is approximately 17 X 13 X 7 inches in size and weighs 28 lbs. It is complete with an optics module, fluidics module, embedded computer, temperature controlled flow cell and touch screen display. The system was originally designed for excitation of an IRD 800 label (excitation maximum 787nm, emission maximum 812nm) and filtered photodiode detection system although it can be modified to be used with an ocean optics spectrometer for spectral detection. In addition, the 785 nm laser diode is easily interchangeable with other compact diode laser sources to allow use with alternate fluorescence labels.

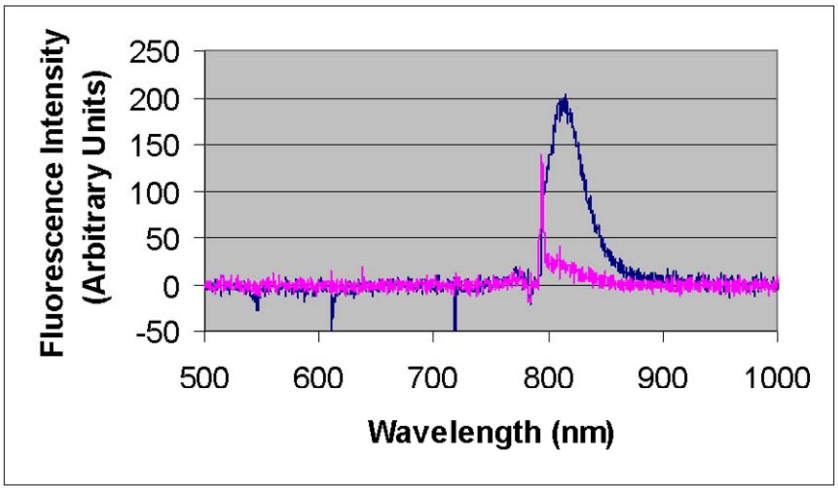

Figure 5. Conventional DNA hybridization experiment using the tapered fiber optic sensor.

Figure 5 shows typical results obtained using the 785 nm excitation of an IRD 800 labeled oligonucleotide in a standard DNA hybridization detection scheme. The darker trace represents the hybridization of e-coli gene *dna K* and the lighter trace is resulting signal after a DDI H2O rinse. These results demonstrate successful binding of oligonucleotides to the tapered fiber optic platform. After the aptamer development is completed, similar experiments will be conducted using the portable DNA detection system and functional aptamer recognition elements in place of standard DNA hybridization used in these studies.

CONCLUSIONS AND FUTURE WORK

We have successfully demonstrated conventional DNA hybridization sensing using the tapered optical fiber platform and have developed a portable detection system for nucleic acid based sensing. Progress to date on the development of aptamer based sensing for the detection of biological agents includes design of a random oligonucleotide library and the development of a PCR amplification method for aptamer selection. Future studies will include the design and development of a molecular aptamer beacon sensing platform. This approach involves integrating the selective molecular recognition capability and high binding affinity of nucleic acid aptamers with the signal transduction methodology of molecular beacons for real-time monitoring of the target. As shown in Figure 6, similar to the conventional molecular beacon, a conformation change occurs in the molecular aptamer beacon (MAB) upon binding to the target, which spatially separates a fluorophore and a quencher to produce a detectable signal.[9] Development of a sensor capable of real-time, species specific identification could dramatically improve the response time to a biowarfare event thereby minimizing or possibly averting casualties.

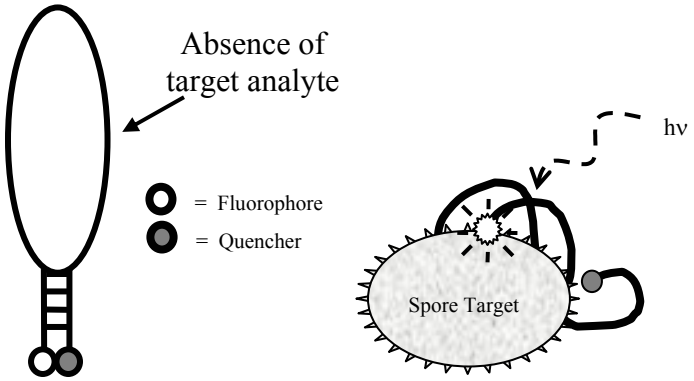

Figure 6. Molecular aptamer beacon sensing concept.

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REFERENCES

1. Iqbal, S. S., Mayo, M. W., Bruno, J. G., Bronk, B. V., Batt, C. A., Chambers, J. P., A Review of Molecular Recognition Technologies for Detection of Biological Threat Agents, *Biosens. Bioelectron,* 15, 549-578, 2000.
2. Stratis-Cullum, D. N., Griffin G D., Mobley J., Vass A. A., and Vo-Dinh T., A Miniature Biochip System for Detection of Aerosolized *Bacillus globigii* Spores, *Analytical Chemistry,* 75, 275-280, 2003.
3. Tyagi, S., Marras, S. A. E., and Kramer, F. R., Wavelength-shifting Molecular Beacons, *Nature Biotechnology* 18 (11), 1191-1196, 2000.
4. Tyagi, S. and Kramer, F. R., Molecular beacons: Probes that Fluoresce Upon hHybridization, *Nature Biotechnology* 14 (3), 303-308, 1996.
5. Belgrader, P., Okuzumi, M., Pourahmade, R., Borkholder D. A., and Northrup M. A., A Microfluidic Cartridge to Prepare Spores for PCR Analysis *Biosens. Bioelectron.* 14 849-852.
6. Jhaveri, S., Rajendran, M., and Ellington, A. D., In vitro Selection of Signaling Aptamers, *Nature Biotechnology* 18 (12), 1293-1297, 2000.
7. Hermann, T. and Patel, D. J., Biochemistry - Adaptive recognition by Nucleic acid Aptamers, *Science* 287 (5454), 820-825, 2000.
8. Sumner, J. J., Mmerole, R. U., Stratis-Cullum, D. N. Yi, H., Bently, W. E., and Gillespie, J. G., Hybridization assay based on evanescent fluorescence excitation and collection, Aerosense 2003 Proceedings.
9. Hamaguchi, N., Ellington, A., and Stanton, M., Aptamer Beacons for the Direct Detection of Proteins, *Analytical Biochemistry* 294 (2), 126-131, 2001.